



Effectiveness of miltefosine-pentoxifylline compared to miltefosine in the treatment of cutaneous leishmaniasis in C57Bl/6 mice

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ABSTRACT

Introduction: The treatment of leishmaniasis is challenging, given the difficulties in drug administration and resistance. Therefore, we chose to test the efficacy of miltefosine combined with pentoxifylline. **Methods:** Twenty-seven isogenic C57Bl/6 mice were infected with *Leishmania (Leishmania) amazonensis*, and equally divided into three groups: miltefosine (200mg/kg/day), miltefosine (200mg/kg/day) with pentoxifylline (8mg/kg/day), and untreated. Response to treatment was evaluated using paw diameter and parasitological criteria. **Results:** The number of viable *Leishmania* reduced significantly within the miltefosine-pentoxifylline group ($p < 0.05$). **Conclusions:** There is hope that a viable treatment exists for *Leishmania* infection.

Keywords: Cutaneous leishmaniasis. Pentoxifylline. Miltefosine.

Cutaneous leishmaniasis (CL) is an infectious disease caused by different species of protozoa from the genus *Leishmania*, and is observed worldwide. The pathogenesis of CL involves a dysregulated immune response with overproduction of proinflammatory cytokines, regulatory cytokines deficiency, and lysis mechanisms by which intracellular parasites escape^{1,2}.

Unfortunately, therapies for treating *Leishmania* infection remain limited, and pentavalent antimonials have remained the drugs of choice for over 60 years. All first and second drug choices, including miltefosine, have high toxicity, require parenteral use, have high rates of relapse and drug resistance, and ultimately lead to treatment failure, especially in immunocompromised patients who desperately require new therapies³. Miltefosine (hexadecylphosphocholine) is an alkylphosphocholine, originally developed for the treatment of cancer, which was discovered to possess antifungal, antiamoebic, and leishmanicidal activity^{4,5}. The drug exerts its cytotoxic effect by interfering with the metabolism of membrane phospholipids, altering the composition, permeability, stability, and fluidity of the cell membrane, subsequently inducing apoptosis^{3,6}. Miltefosine's mechanism of action also indirectly

induces immunomodulatory antileishmanial mechanisms by increasing production of gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), and interleukin 12 (IL-12), thereby increasing phagocytosis and inducing an immune response biased towards a Th1 profile^{7,8}.

Similarly, pentoxifylline (a methylxanthine) has been investigated in the treatment of CL, based on its inhibition of nuclear factor kappa beta (NF- κ B), TNF- α , and intercellular adhesion molecule 1 (ICAM-1)^{8,9}, as well as its nephroprotective effect when combined with antimonials¹⁰. These immunomodulatory effects may help to regulating patients' exacerbated and inefficient intracellular immune response to parasites, and subsequently reduce tissue inflammation. Therefore, this study aims to examine the combined immunomodulatory effects of two oral drugs, by comparing miltefosine-pentoxifylline to miltefosine alone.

In this study, we used 27 isogenic C57Bl/6 mice and promastigotes of *Leishmania (Leishmania) amazonensis* (MHOM/BR/PH8) from the Dermatologicology Laboratory at the University of Brasília. All mice were born and raised in the vivarium of the Faculty of Medicine at the University of Brasília. This species was chosen since it is a model parasite that grows easily in culture media, improving study speed and viability. While it is not the most prevalent species, *Leishmania amazonensis* is distributed throughout the country, where it causes severe clinical manifestations and diffuse cutaneous leishmaniasis. No satisfactory treatment has been discovered¹¹.

These experiments were evaluated and approved by the ethics committee for animal use in the Institute of Biological Sciences of the University (UnBDOC 43044/2010). All animals

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were inoculated with 10^7 parasites in metacyclic promastigote form, which were injected subcutaneously in the dorsal tissue of the right footpad, following the standardized technique⁴. Thirty days after inoculation, the mice were divided according to sex (6 females and 21 males) and randomly sorted into 3 groups, each group containing 2 females and 7 males. The groups were labeled and treated as follows: (M) oral miltefosine (200mg/kg/day), (M + P) oral miltefosine (200 mg/kg/day) with oral pentoxifylline (8mg/kg/day), and (C) no treatment. Oral dosage was performed daily for a period of 12 days, using a gavage needle. Treatment response was evaluated using the lateral diameter of the right paw, lymph culture in Novy-MacNeal-Nicolle (NNN) media, the methylthiazol tetrazolium (MTT) dye colorimetric assay, and visual detection of amastigotes.

We measured the lateral-lateral diameter of the right footpad of each mouse, using a Mitutoyo® millimeter caliper, on the first (D1), sixth (D6) and twelfth (D12) day of treatment. On the thirteenth day (D13), lymph was aspirated from the infected paws and cultured in NNN media. Cultures were observed daily, under an inverted light microscope at 40× magnification, to detect the presence of parasites. The MTT test was carried out using a limiting dilution assay as previously described¹². In short, the infected paws of 2 male mice from each group were subjected to sterile dissection and trituration in 5ml of Schneider's culture medium, enriched with 20% fetal bovine serum. Serial dilution was performed in standard 96 well plates. Plates were incubated at 26°C for 4 days, with daily observation under an inverted light microscope. At the end of that period, plates were subjected to the MTT colorimetric assay, as previously described¹³. Each culture assay was performed at the end of treatment (D13) and on D63. On D13, group (C) consisted of 7 mice, group (M) 7 mice, and group (M + P) 8 mice. On D63, group (C) consisted of 5 mice, group (M) 3 mice, and group (M + P) 4 mice.

Statistical analysis: Statistical analysis was performed using R 3.0.0 software (R Core Team, 2013).

Ethical considerations: From the original 27 mice, 9 died prior to the end of the experiment, mainly due to infighting and resulting injuries. Six mice per group were considered adequate for statistical analysis, and the possibility of animal death had been considered at the time of project approval by the Ethics Committee (UnB-Doc 43044/2010).

Paw diameter: Since paw diameter was not normally distributed (based on the Shapiro Wilk and Kolmogorov-Smirnov tests), we used the Kruskal-Wallis test (using a chi-square distribution) to analyze the three groups' response to treatment. Our analysis found that on D1, the treatment effect was not significant ($p = 0.06$). However, the treatment effect on D6 and D12 was significant ($p < 0.01$, $p < 0.01$). Since the sample size was small, we tested the suitability of the Kruskal-Wallis test using Monte Carlo tests. Unfortunately, we were unable to process a sample calculation for each variable in the analysis. Although the p -values were different between the two methods, the inferences were always consistent, so we concluded that use of the asymptotic method did not cause bias in the inference.

Since the Kruskal-Wallis test found no difference between (M) and (M + P) on D6 and D12, we investigated the differences in pairs, using the Wilcoxon test to analyze the groups head-to-head. Treatment with both (M) and (M + P) significantly reduced paw diameter compared to the control group, although neither group was found to be superior (**Figure 1**). The respective p -values are shown on **Table 1**. Dunn's posttest was applied after the above analysis, and the same inferences remained.

MTT colorimetric assay: The absorbance values obtained from the first row of wells (line A) of the plates (corresponding to the undiluted *Leishmania* concentration from the paws) were used for comparing the groups, using the Wilcoxon test. Our results (**Figure 2**, **Table 1**) indicate that there was a significantly lower concentration of *Leishmania* in the (M + P) group, compared to the (M) group.

Analysis of cultures: Cultures obtained on D13 were positive in all 6 samples from group (C), indicating the presence of infection. In group (M), 1 of 6 cultures was positive, and in group (M + P), 2 of 6 were positive. Fisher's t-test only detected a significant difference between group (C) and (M) (**Table 1**).

Analysis of slides: Parasites could only be detected visually in the control group (C). All slides from the other groups showed no signs of parasites.

We chose not to use N-methyl glucamine (the first line of treatment for *Leishmania* infection), since we know that miltefosine has the same efficacy in *Leishmania amazonensis*⁴, and our goal was to test whether a combination of oral drugs would provide a better response to treatment. When we analyzed the paw diameters, we adopted a significance (alpha) level of 5%, and both treatment groups (M, M + P) were significantly smaller ($p < 0.05$) than group (C) on D6 and D12. However, there was no significant difference between the two treatment groups, and thus there is no evidence that either is superior. These results may indicate that a longer period is required paw

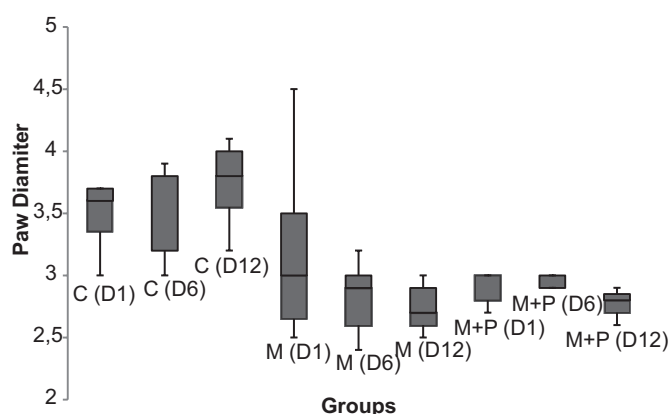


FIGURE 1 - Paw diameter over time. Paw diameters were measured at three time points (D1, D6, and D12). D1 represents diameters before initiating treatment, while D6 and D12 represent diameters on the sixth and on the twelve day of treatment. M: miltefosine; M+P: miltefosine plus pentoxifylline; C: control.

TABLE 1 - Analysis of experimental parameters.

Parameter of analysis	Group C	Group M	Group M + P	Time	Groups	Average number*	p-value
Diameter of the paws (mm)	3.444 ± 0.302	2.855 ± 0.254	2.855 ± 0.194	D6	C vs M		<0.01
					C vs M + P	7.33	<0.01
					M vs M + P		0.92
	3.742 ± 0.315	2.766 ± 0.176	2.733 ± 0.124	D12	C vs M		<0.01
					C vs M + P	7.33	<0.01
					M vs M + P		0.65
Absorbance of A line	1.074 ± 0.198	0.406 ± 0.103	0.221 ± 0.110	D13	C vs M		<0.01
					C vs M + P	7.33	<0.01
					M vs M + P		<0.01
	1.446 ± 0.146	0.381 ± 0.045	0.182 ± 0.036	D63	C vs M		<0.01
					C vs M + P	4	<0.01
					M vs M + P		<0.01
Positive cultures (n)	6	1	2	D13	C vs M		0.01
					C vs M + P	7.33	0.06
					M vs M + P		0.99

C: control group; **M:** miltefosine group; **M + P:** miltefosine plus pentoxifylline group; **D:** days after initiating treatment; **vs:** versus. *Average number of animals in the three groups.

diameter to indicate healing and regression of local edema and infiltration, even in the absence of viable parasites. Secondary infections and local trauma caused by the mice's infighting also cannot be discarded.

The MTT colorimetric test indirectly assesses cellular mitochondrial activity and cellular viability, and is relatively immune to observer bias. The absorbance value increases with higher concentrations of formazan (a product of mitochondrial MTT conversion)¹⁴, and since there is no cutoff point, a direct

comparison between the effects of the two treatments can be made. Although our primary goal was to determine which of the treatments eliminated the largest number of parasites, we chose not to monitor the mice until the absolute disappearance of parasites and lesions, since the resulting experiment would be burdensomely long. We found that combined treatment (M + P) was more effective than (M) alone, when both were compared to group (C), as evidenced by the lower absorbance values. Therefore, the combination of miltefosine and pentoxifylline provided greater leishmanicidal effect than miltefosine monotherapy. The greater effectiveness of the combination treatment was also observed D63, given the persistent leishmanicidal effect, although the sample size was suboptimal (**Table 1**).

The visual observation of the slides did not concretely contribute to the conclusions of this experiment, as visualization of the amastigotes on the slides was inaccurate, possibly caused by low sensitivity or observer error. However, when the cultures were analyzed, we only observed a significant difference ($p < 0.05$) between (C) and (M). Such an inference contradicts the results our absorbance data, which is more accurate than direct observation. Therefore, various factors, such as contamination bias, sensitivity of cultures (30-50%), and the number of repetitions of each culture, should have been more adequately addressed, since they may have contributed to inadequate observation measurements. The results also could have been validated by histopathological evaluation, although this technique is less sensitive than other imaging techniques, since the cellular infiltrates can persist for years after treatment as previously described.

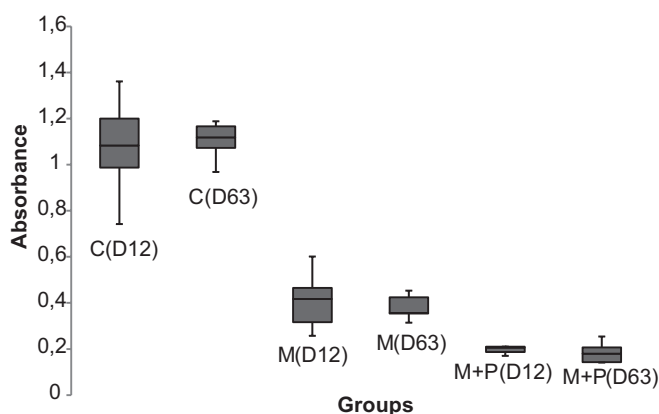


FIGURE 2 - Methylthiazol tetrazolium absorbance. **D:** days after initiating treatment; **C:** control; **M:** miltefosine; **M+P:** miltefosine plus pentoxifylline.

The use of drugs with immunomodulatory effects to treat CL is primarily motivated by the need for alternatives to the standard treatment (pentavalent antimony), and has shown promising results in relapse cases^{8,15}. The use of combined pentoxifylline-miltefosine treatments could help reduced drug resistance to miltefosine, as well as possibly increase the efficacy of treatment⁸. Pentoxifylline has also been shown to have a nephroprotective effect when combined with pentavalent antimonials¹⁰.

A treatment scheme, preferably using oral administration, is necessary, especially in Brazil, where CL is common in the North and Northeast regions. Treatment is especially critical in the Northeast region, which is plagued by drought, extreme poverty, and limited access to healthcare. Considering the high rates of CL resistance to treatment, our results provide hope that combining drugs with antileishmanial and immunomodulatory effects can be effective in combating microbial resistance, as observed in the multidrug regimens used to treat leprosy and tuberculosis.

Our experiments demonstrate the superiority combined miltefosine and pentoxifylline in the colorimetric assay, which is the most accurate test for evaluating treatment efficacy. Therefore, we conclude that this combination may be more effective than the use of miltefosine alone, in the treatment of CL due to *Leishmania amazonensis* in C57Bl/6 mice. From a statistical point of view, there are no factors that would invalidate the results. However, we did not evaluate the power of the tests, since we were required to terminate the experiments due to ethical considerations. Therefore, it is necessary to perform more tests, using other *Leishmania* species prevalent in Brazil, such as *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) guyanensis*, to confirm and expand these results.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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